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Mechanisms of Free Radical Chemistry and Biochemistry of Benzene

by Lisa R. Karam* and Michael G. Simic*

o-Tyrosine (o-Tyr) was used as a specific biomarker for ·OH radicals generated in biosystems. Specificity of o-Tyr as an ·OH biomarker was based on previous studies in systems exposed to ionizing radiations. Fresh muscle tissue incubated with benzene for 1 hr at 38°C exhibits formation of o-Tyr as seen in the cases of ethanol- and carbon tetrachloride-exposed systems. Gas chromatography/mass spectrometry selective ion monitoring measurements of o-Tyr yields in chicken breast muscle incubated with water or benzene indicate levels of less than 0.1 ppm and 3.0 ± 0.5 ppm of o-Tyr, respectively. Formation of ·OH is presumed to originate via a Haber-Weiss reaction of H_2O_2 with Fe (II) preceded by the formation of $\cdot O_2^-$ and H_2O_2 from distorted mitochondria.

Introduction

Hydroxy derivatives of benzene such as phenol and hydroquinone (*1*) are catabolic products in benzene-exposed biosystems. In addition, benzene can give hydroxy derivatives *via* free radical processes (*2*). Therefore, it is important to distinguish between catabolic and free radical processes in order to understand the mechanisms of benzene toxicity.

A convenient approach in the study of free radical processes is through the use of radiation techniques, such as pulse radiolysis and gamma radiolysis (*2*). Gamma rays decompose water into free radicals:

$$H_2O \xrightarrow{\checkmark} \cdot OH\ (2.8) + e^-_{aq}\ (2.8) + H\ (0.6) \quad (1)$$

where the numbers in parentheses indicate G-values (number of species formed per 100 eV of absorbed energy).

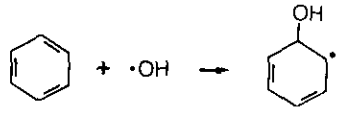
The major interest in previous studies of the radiation chemistry of protein substituents (e.g., amino acids) in aqueous solution has been very often ·OH radicals and their reactions. To optimize the yields of these radicals and to study their reactions specifically, the hydrated electron can be conveniently converted into ·OH through interaction with nitrous oxide (N_2O):

$$e^-_{aq} + N_2O \longrightarrow \cdot OH + OH^- + N_2 \quad (2)$$

Hence,

$$H_2O\ (N_2O) \xrightarrow{\checkmark} \cdot OH\ (5.6) + H\ (0.6) \quad (3)$$

The hydroxy radical is very reactive (*4*) and reacts with most biocomponents. With benzene it was shown to add readily to the ring.



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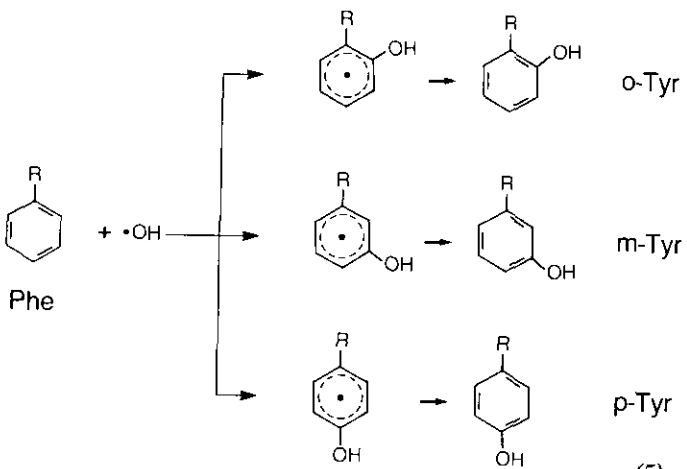
The intermediate free radical is an hydroxycyclohexadienyl radical (*5*), which leads to the formation of phenol both in the presence and in the absence of oxygen. The transient free radical can be observed easily by pulse radiolysis (*5,6*).

This work deals with the effect of benzene on muscle tissue *via* the generation of free radicals. The work is an extensive of previous observations that demonstrated the formation of ·OH radicals in muscle tissue by ethanol and carbon tetrachloride (*7*). Since catabolism of benzene also gives phenol under normal biological conditions, Equation 4 is not suitable as an indicator of generator of ·OH radical. Therefore, an alternate biomarker was required in

*Center for Radiation Research, National Bureau of Standards, Gaithersburg, MD 20899.

Address reprint requests to L. R. Karam, Center for Radiation Research, National Bureau of Standards, Gaithersburg, MD 20899.

order to determine the presence of ·OH. In this work, *o*-tyrosine (*o*-Tyr) resulting from the reaction of ·OH radical with phenylalanine (Phe) (*8*) is used as an indicator of ·OH presence in a biosystem (*7,9*). The mechanism of *o*-Tyr formation is shown below:



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Methods

Irradiations. Pieces of fresh chicken breast meat (approximate gross weight of each piece, 50 g) of cubic shape was irradiated in a ^{60}Co-gamma source (dose rate, 19.85 krad/min, dose range 0.05–8.0 Mrad) in the presence of air and at ambient temperature. Samples were then dried *in vacuo*.

Separation of Muscle Fiber. After irradiation, water-soluble muscle fraction was removed by three successive water (1 mL) extractions of the dried and pulverized samples.

Incubation. Pieces of fresh (1 hr from slaughter) chicken breast meat or of lean and tender backbone beef (approximate weight of each piece, 50 g) were diced and incubated with either 25 mL of water or of benzene for 1 hr at 38°C in the presence of air and the absence of light. Samples were dried *in vacuo*.

Hydrolysis. The dried samples were pulverized and 5 to 10 mg of powder were hydrolyzed with 6 N HCl for 6 hr at 150°C in sealed and evacuated tubes. *p*-Hydroxyphenylglycine (1.00×10^{-9} mole) was added to each sample prior to hydrolysis as an internal quantitative standard.

Derivatization. Hydrolyzed samples were dried *in vacuo* and then derivatized with a mixture of acetonitrile and *bis* (trimethylsilyl) trifluoroacetamide (BSTFA) (1:2) at 130°C for 30 min under N_2 in Teflon-capped hypovials.

Analysis. Derivatized samples were separated on a fused silica capillary column (25 m, 0.2 mm ID) coated with cross-linked 5% phenylmethylsilicone, siloxane deactivated (film thickness, 0.11 μm) from Hewlett-Packard using a temperature program of 100°C to 250°C at a rate of 10° C/min. Analyses were undertaken using the selective ion monitoring (SIM) technique with a Hewlett-Packard Model 5970A mass selective detector interfaced to a Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph. The injection port was maintained at 250°. Characteristic ions of *o*-Tyr TMS derivative fragmentation were measured at m/z 397, 382, 354, and 280. All *o*-Tyr values are an average of at least four measurements and two animals.

Results and Discussion

The yield of *o*-Tyr in fresh muscle tissue incubated with water or benzene is shown in Table 1. The *o*-Tyr yield is expressed in parts per million per dry protein because of possible variability of water content in meats. Neither benzene nor its radicals can oxidize the benzene ring to generate tyrosines because of the high stability of the benzene ring (*6*). The fact that no detectable *o*-Tyr is found in water-treated meats further indicates no enzymatic formation *o*-Tyr.

When ·OH radicals were generated by ionizing radiation (gamma-rays), *o*-Tyr was found to increase linearly with the dose (Fig. 1). Ionizing radiations split water (~70–76%) in the muscle tissue into ·OH radicals (*7*) (Eq. 1), which react with most subcomponents of the biomaterial. Because of its high reaction rate with ·OH and reasonably high abundance (~2–4% in muscle tissue), phenylalanine is a predominant reactant. If one calculates the yield of ·OH radicals in irradiated muscle (chicken breast), one finds that approximately 1 ·OH radical out of 15 has reacted with Phe (*o*-Tyr is about one-third of the total tyrosine yield). This yield is also expected from the abundances and ·OH reactivities of all 20 amino acids (*4*). Assuming similar distribution of radicals, the yield of *o*-Tyr in benzene-treated chicken breast muscle (3 ppm in dry protein) (Table 1) is equivalent to approximately 3 kGy (0.3 Mrad) (Fig. 1), or 0.68 mmole *o*-Tyr/kg.

Examples of the SIM chromatograms utilized in the measurement of *o*-Tyr in chicken breast meat appear in Figure 2 for the three irradiation dose points 0, 5 and 80 kGy (0, 0.5, and 8.0 Mrad). Chromatograms from benzene-incubated chicken are similar.

Hydrogen peroxide is generated in most cells and is also present in body fluids as a consequence of oxygen metabolism. Under normal conditions, catalase successfully eliminates H_2O_2. However, when greater than average quantities are generated and the biocomplexes and

Table 1. Comparision of *o*-Tyr yields in chicken breast meat.[a]

H_2O incubated	Benzene incubated	Carbon tetrachloride incubated	Ethanol-incubated	Irradiated, per Mrad
< 0.1 ppm	3.0 ± 0.5 ppm	5.1 ± 0.9 ppm	2.5 ± 0.4 ppm	12 ± 2 ppm

[a]Each value is the mean of at least four measurements each in two animals ± one SD. Experimental details appear in the text.

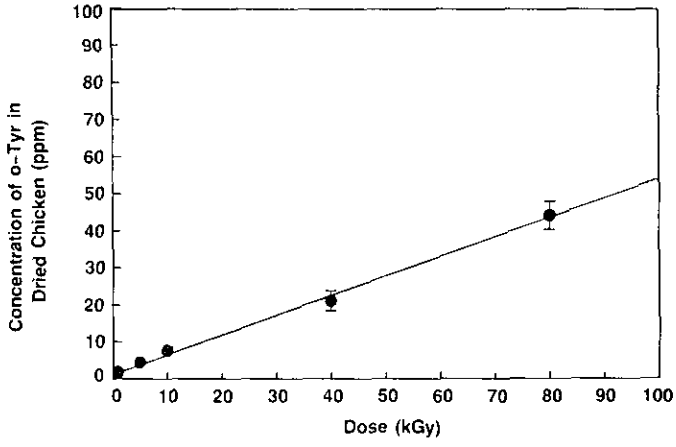


FIGURE 1. Yield of *o*-Tyr (in ppm) in irradiated chicken breast muscle fiber. *p*-Hydroxyphenylglycine was used as an internal standard. Calculations were based on SIM results. Experimental details appear in the text.

cellular substructures (e.g. mitochondria, cellular membranes) are perturbed, it is possible that a Haber-Weiss (*10*) reaction is favored:

$$H_2O_2 + Fe(II) \longrightarrow {}^{\bullet}OH + OH^- + Fe(III) \qquad (6)$$

Such a reaction has been postulated to occur in biosystems (*11-13*), but no direct proof has been available.

The presence of *o*-Tyr, a specific biomarker for $^{\bullet}OH$ radicals, in muscle tissue exposed to benzene demonstrates unequivocally that the $^{\bullet}OH$ radical can be generated in benzene-stressed biosystems. Stressed mitochondria have been shown to generate $^{\bullet}O_2$ radical anions *via* electron transfer from semiubiquinone to oxygen (*14-16*). This process is expected to lead then to the formation of large quantities of hydrogen peroxide (*11*). Since, in general, biosystems contain negligible amounts of free Fe(II), hydrogen peroxide will be converted into $^{\bullet}OH$ radical only in the presence of appropriate Fe(II) complexes. In view of the relatively large levels of H_2O_2 in blood plasma (micromolar concentrations), one can surmise that such reactive Fe(II) complexes are not very abundant. Incubation of muscle tissue with organic solvents may, in addition to generation of hydrogen peroxide, alter the Fe(II) complexes in such a way as to make them more reactive with hydrogen peroxide. This process can be summarized by:

$$H_2O_2 + Fe(II)Bio \xrightarrow[k = \text{very slow}]{} \text{negligible} \qquad (7)$$

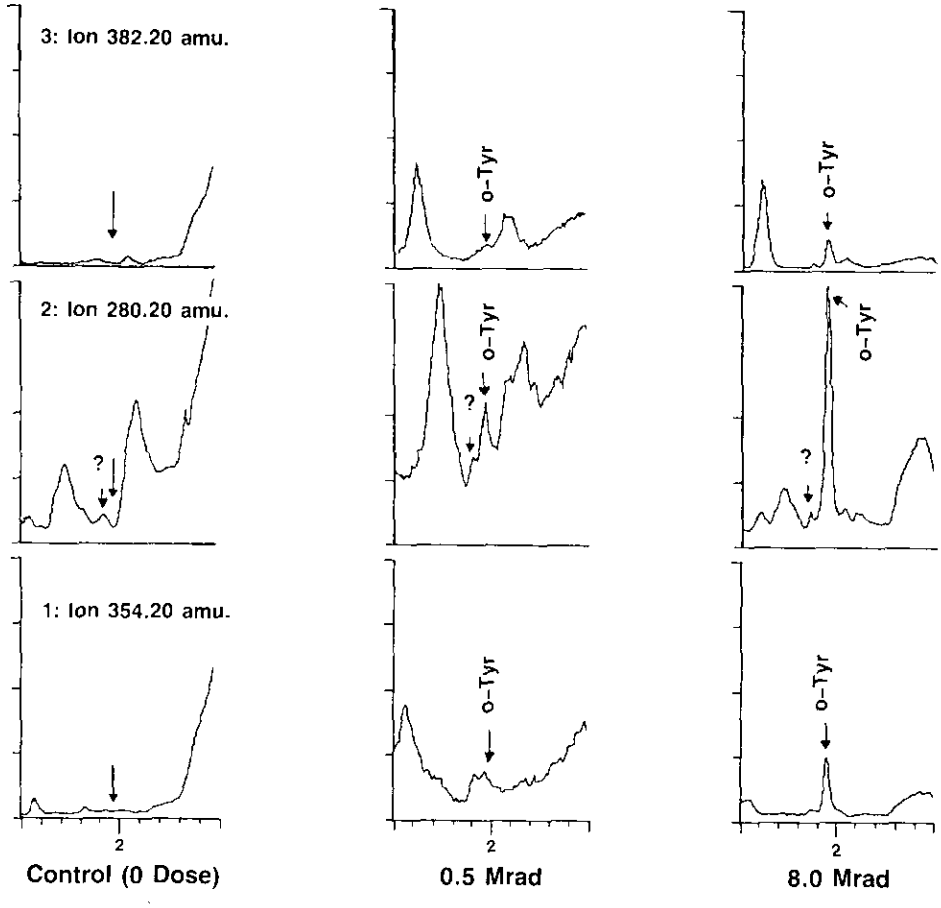


FIGURE 2. SIM chromatograms of irradiated and dried chicken breast samples. Samples analyzed after HCl hydrolysis and trimethylsilylation. *o*-Tyr ions followed: m/z 382.20 $(M-15)^+$, m/z 280.20 $(M-117)^+$, m/z 354.20 $(M-43)^+$. Experimental details appear in the text.

$$\text{Fe(II)Bio} \xrightarrow[\text{solvent}]{\text{organic}} \text{Fe(II)Bio}' \quad (8)$$

$$H_2O_2 + \text{Fe(II)Bio}' \xrightarrow[k = \text{high}]{} {}^{\bullet}OH + OH^- + \text{Fe(III)Bio}' \quad (9)$$

Once the levels of ·OH radical in tissue or tissue culture are determined, the discrimination of free radical processes and their consequences from metabolic processes and their consequences is possible. This is particularly relevant in the case of benzene metabolites, phenol and hydroquinone, since ·OH radical reaction with benzene gives the same products.

Phenol (Ph-OH) is generated from hydroxycyclohexadienyl radicals in the presence and absence of oxygen. In the absence of oxygen, stronger oxidants, including the radical itself, oxidize the radical to phenol

$$\bullet\text{PhH-OH} + \text{Ox} \longrightarrow \text{Ph-OH} + \text{Red} \quad (10)$$

or

$$\bullet\text{PhH-OH} + \bullet\text{PhH-OH} \longrightarrow \text{Ph-OH} + \text{PhH}_2\text{-OH} \quad (10a)$$

$$\longrightarrow \text{HO-PhH-PhH-OH}$$

$k = 7 \times 10^8\ M^{-1}\ s^{-1}$ [Ref. (6)] (10b)

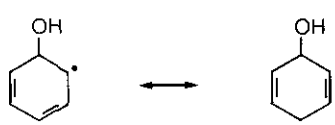
The dimer is unstable and loses water to give biphenyl (*17*)

$$\text{HO-PhH-PhH-OH} \longrightarrow \text{Ph-Ph} + 2H_2O \quad (11)$$

In the presence of oxygen, the ·OH adduct to benzene reacts with oxygen

$$\bullet\text{PhH-OH} + O_2 \longrightarrow \bullet\text{OOPhH-OH} \quad (12)$$

Since ·PhH-OH has two mesomeric forms



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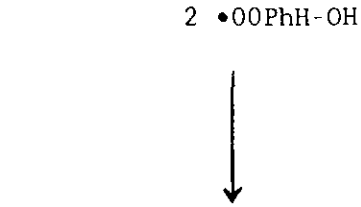
there will be two types of peroxy radicals formed in Equation 12. The reaction sequence for both forms is similar,



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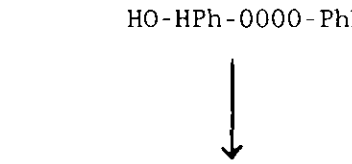

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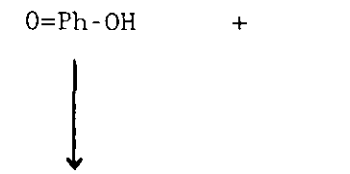

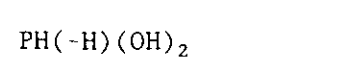

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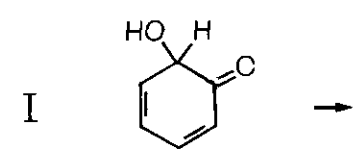
The Ph(-H)$(OH)_2$ is a multiple product since O=Ph-OH has two forms as indicated below.



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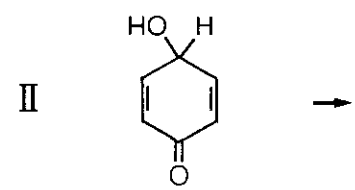

(18)

Hence both catechol and p-hydroquinone should be generated in oxygenated systems.

While *o*-Tyr is not a specific product from the metabolic pathways of benzene, its presence indicates the formation of ·OH. In systems exposed to benzene, these radicals must arise through some interaction of the benzene molecule with the biological matrix.

Conclusions

o-Tyr is a product of the interaction of ·OH radicals with Phe in the presence and absence of oxygen. Since it is formed only through the generation of free ·OH, it is used as a specific biomarker for ·OH radical. *o*-Tyr yields in biological systems (in this case, chicken breast meat) exposed to benzene indicate that the amount of ·OH radicals generated in such a stressed system is very high. Compared to radiation, benzene-induced ·OH rad-

icals are equivalent to a radiation dose of approximately 3 kGy (300 krad). The observed phenomenon is a consequence of the effects induced by an organic solvent. Aqueous solutions of benzene (20mM at saturation) are not expected to generate ·OH radicals. However, metabolism and biological concentration of benzene may lead to alterations in the electron transport chain and subsequent formation of ·OH.

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